PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
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Donald W. KUFE

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For: MODULATION OF INTERACTION OF MUC1 WITH MUC1 LIGANDS

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CERTIFICATE OF ELECTRONIC TRANSMISSION

I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:

October 13, 2011
Date

Steven L. Highlander

SECOND INVENTOR'S DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

I, Surender Kharbanda, do declare that:

1. I am the Surender Kharbanda named as an inventor on the above-captioned application.

2. My *curriculum vitae* has been submitted with my previous declaration.

3. In my previous declaration, I reported on the creation of MUC1-C-ED-Fc chimeric proteins [Glycosylated (GO-101) and Unglycosylated (GO-102)]. Human MUC1-C-ED (Figure 1) was fused with the PCR-amplified mouse or human IgG Fc fragment and cloned into the pCR3.1 vector between NheI and XbaI sites. The pCR3.1 mFc-MUC1-C-ED or pCR3.1 hFc-MUC1-C-ED plasmids were stably transfected into the Chinese Hamster Ovarian (CHO) cell line CHO-K1. Separately, these plasmids were also transfected into mutant Lec1-CHO cells, which lack GlcNAc glycosyl transferase so that N-linked carbohydrates are blocked at the Man5-GlcNAC2-Asn intermediate. This cell line is used to produce the unglycosylated mFc-MUC1-C-ED and hFc-MUC1-C-ED chimeric proteins. These stable cell lines were grown in BD cell monoclonal antibody production chambers (Cell line 1000) in serum free media. Secreted proteins in the supernatant were passed through protein-A column, washed extensively and eluted with 0.1 M Citric acid, pH 3.0. The eluted proteins were concentrated and run on SDS-PAGE gels to confirm size and purity. The human Fc-MUC1-C-ED glycosylated protein is designated as GO-101 and unglycosylated protein is designated as GO-102.

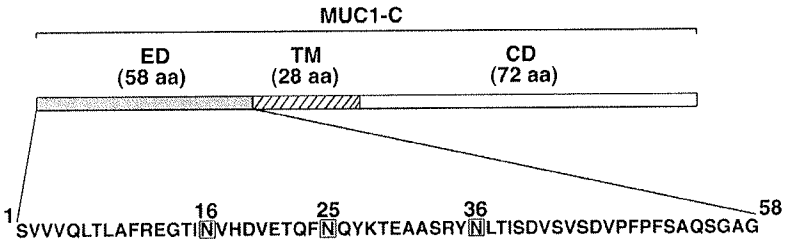


Figure. 1. *Schematic representation of the transmembrane MUC1-C subunit and amino acid sequence of the extracellular domain (MUC1-ED).*

4. My previous declaration also reported the *in vitro* inhibitory effects of GO-101 on various cancer cell lines, as well as an *in vivo* experiment with hormone-dependent ZR-75-1 human breast carcinoma cells in a tumor xenograft model.

5. I now report additional studies that have been completed since the previous submission. First, to assess the effects of GO-101 on sensitivity of human carcinoma cells to oxidative stress, we treated ZR-75-1 breast carcinoma cells with 500 nM GO-101 in the presence and absence of 1.0 mM H_2O_2. As a control, cells were also separately treated with hFc (data not shown). Following treatment for 3 days, viable cells were then counted using trypan blue exclusion. The results demonstrate that ZR-75-1 cells exposed to H_2O_2 exhibited ~66% inhibition in cell proliferation compared to mock conditions. Importantly, the ZR-75-1 cells exposed to 500 nM GO-101, but not hFc, was associated with ~42 % growth inhibition. However, there was a ~90% growth inhibition when cells were treated with both H_2O_2 and GO-101 (Fig. 2).

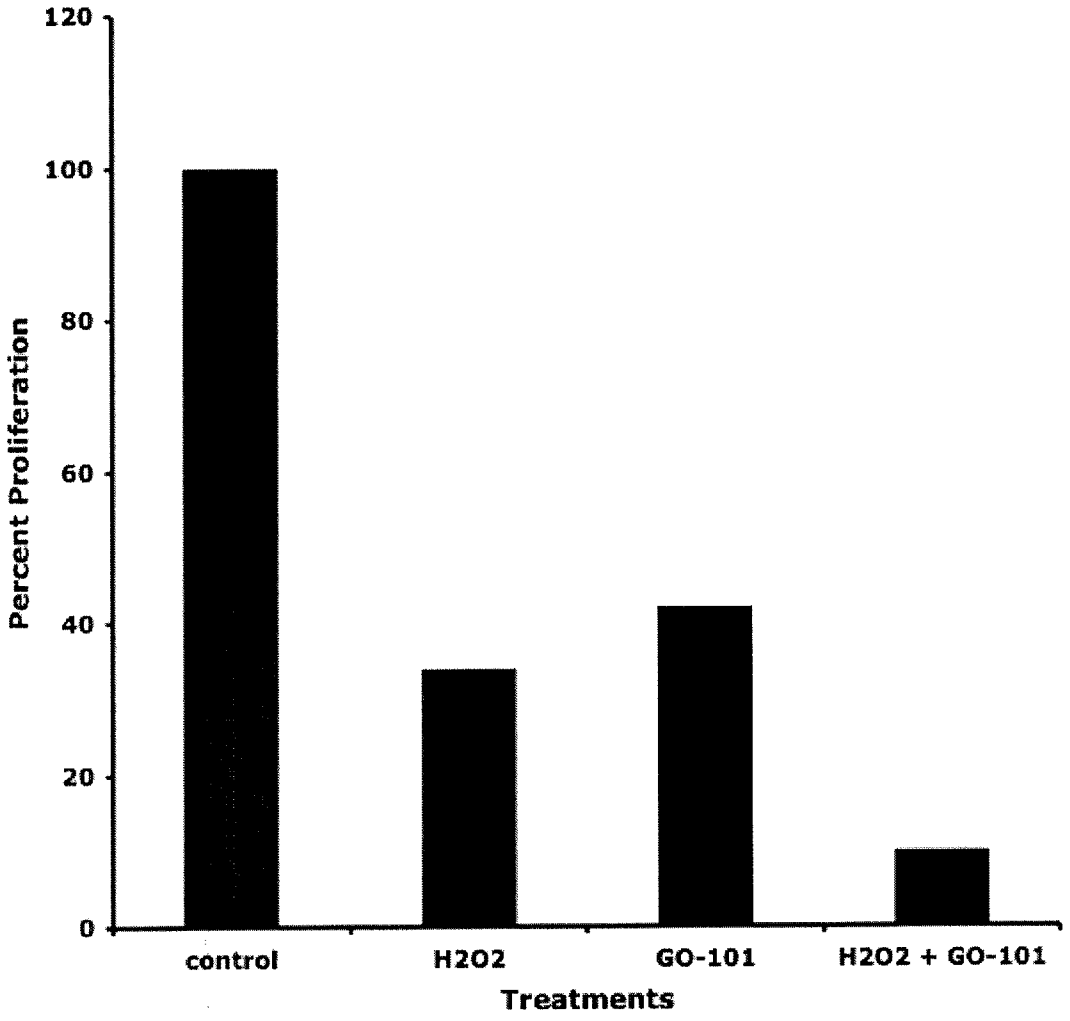
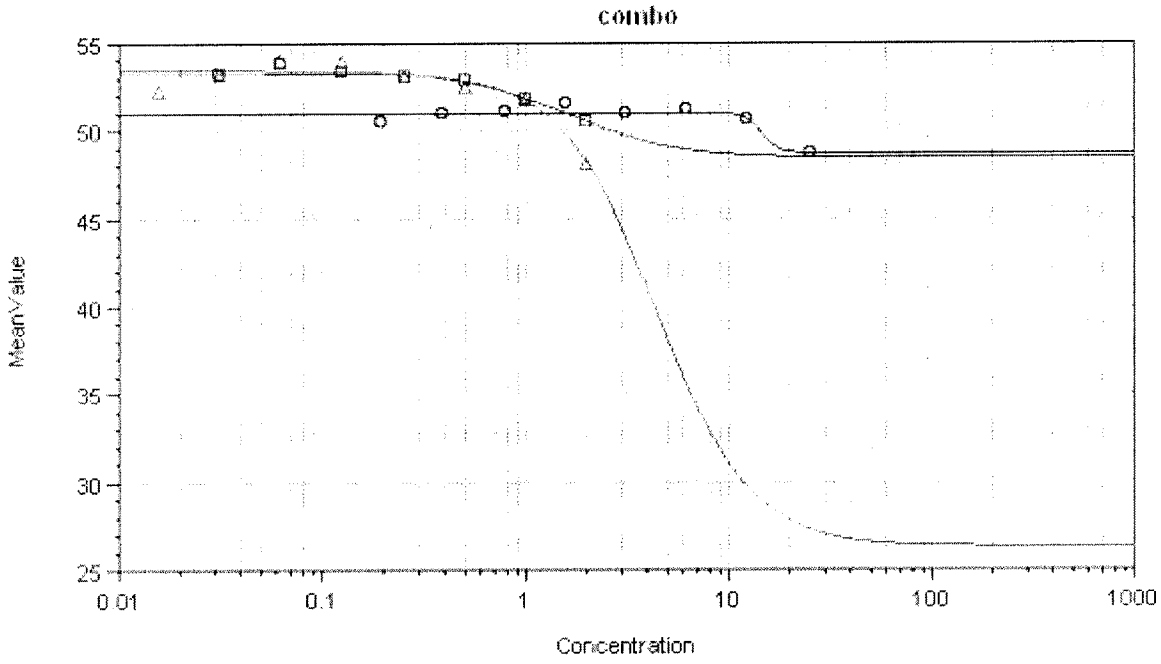


Figure 2. *ZR-75-1 human breast carcinoma cells were treated with 500 nM GO-101 and/or 1 mM H2O2 for 3 days and the cell proliferation was measured by trypan blue exclusion method. The percent proliferation is shown to that compared with control.*

6. ZR-75-1 human breast carcinoma cell were treated with various concentrations of GO-101 starting from 2 μM with 2-fold serial dilutions to obtain a total of 8 different concentrations in the presence or absence of doxorubicin starting from 25 nM with 2-fold serial dilutions to obtain a total of 8 different concentrations. Cells were also separately treated with multiple concentrations of doxorubicin starting from 25 nM with 2-fold serial dilutions to obtain a total of

8 different concentrations. Cells in duplicate wells were treated with either alone (doxorubicin or GO-101) or in combinations for 4 days. On day 5, the medium in the plate was replaced with 10% alamarBlue solution and incubated for various time points (1-5 hrs). At the end of each hour, absorbance of the plate was measured at 570 nm and 600 nm as reference. A set of blank wells were maintained with medium alone and medium with alamarBlue for the purpose of calculating actual absorbance. Obtained absorbance values were plotted against respective concentrations of GO-101, doxorubicin or in combination of GO-101 and doxorubicin The results demonstrate that there was a very little inhibition of cell proliferation at low doses of either agent alone. However, there is a substantial reduction in cell proliferation when the two agents combined. Fig. 3 indicates a representative plot obtained for one of the assays performed with these conditions.



	y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
o	Doxo 25 nm (Doxo 25 nM: Concentration vs MeanVa...	51.035	10.337	14.543	48.723	0.881
△	combo (Combo: Concentration vs MeanValue)	53.343	1.851	4.369	26.29	0.914
□	p59 (3-27-10) (p59 (3-27-10): Concentration vs Mea...	53.468	1.591	1.601	48.433	0.969

Figure 3: ZR-75-1 breast carcinoma cells in a 96 well plate were treated with various concentrations of purified of GO-101 (p59, red curve), multiple concentrations of Doxorubicin (blue curve) and combinations of GO-101 and Doxorubicin (green curve) for four days. AlamarBlue dye was added to the cells on day 5, and the absorbance was measured at 570 and 600 nm. A 4-parameter curve was obtained by plotting the percentage reduction of alamarBlue calculated using these absorbance values against respective treatment concentrations.

7. As mentioned above, the production of GO-101 protein using CHO-K1 stably transfected with pCR3.1 hFc-MUC1-C-ED vector is very low. Hence, we have performed GO-101 and doxorubicin combination study by using only one low dose of GO-101.

Study Protocol. GO-101. To avoid freeze/thaw cycles, GO-101 protein (HC-batch GENU001) was prepared as multiple aliquots of frozen solution in PBS into individual treatment doses. The frozen tubes were stored at -80°C until initiation of dosing. On each day of treatment, the appropriate tubes were allowed to thaw at room temperature. The resulting solution in each tube was clear and colorless with a pH of ~ 6.5.

Doxorubicin. 2 mg/ml doxorubicin (lot # 1705942, CRL/MIR) was manufactured by Bedford Labs and obtained from McKesson Speciality Care Solutions. Upon receipt, the clear, red solution was stored at 4°C, protected from light. Dosing solutions were prepared just prior to each treatment by dilution with saline.

Nu/nu mice. About 9-10 weeks old female mice (on the day of treatment) (CRL: NUFoxn1nu) were obtained from Charles River Labs. The mice were fed irradiated Rodent Diet 5053 and water *ad libitum*. All the treatments, body weight determinations and tumor measurements were carried out in the bubble environment. Test animals were implanted

subcutaneously, high in the right axilla, on Day 0 with 5 x 10^6 cells/animal (0.2 ml) using a 27 gauge needle and syringe.

Tumor cells. ZR-75-1 cells were obtained from ATCC and expanded using RPMI 1640 media as described above. ZR-75-1 human breast carcinoma is an estrogen-dependent tumor model, and all of the mice were implanted with 17-β estradiol pellets (0.36 mg/pellet, 60 day release, Innovative Research of America). The pellets were implanted subcutaneously on the back of the neck with a 10-gauge trocar needle prior to tumor implant. Blood levels of 17-β estradiol were not monitored during the experiment.

Dosing. All animals dosed with either doxorubicin alone or in combination with 1 mg/kg GO-101 daily x 21. Doxorubicin was injected 6 mg/kg i.v. Q4D x 3 in 200 µl volume and GO-101 was injected with a fixed volume of 100 µl as shown in Table 2.

Table 2

Groups	Compound	# of Mice	Route	Schedule	Dose (mg/kg)
1	Doxorubicin	5	iv	Q4D x 3	6 mg/kg
2	GO-101+Dox	5	i.p./i.v	QD x 21	1 mpk + 6 mpk

Results. Treatment with doxorubicin at 6 mg/kg given every fourth day for three injections plus GO-101 (1 mg/kg, daily x 21) was apparently tolerated, producing no "treatment-related" toxicity. Treatment with doxorubicin produced significant ($p = 0.0356$) anti-tumor activity based upon tumor growth delay. Doxorubicin treatment produced neither tumor regressions nor tumor free survivors. Combination treatment with doxorubicin + GO-101 produced a significant inhibition of tumor growth to that compared with Doxorubicin alone (Fig. 4). Two out of 5 animals in this group had complete tumor regressions. Although this treatment

regimen appeared to produce more tumor regressions compared to single agent therapy, suggesting enhanced anti-cancer activity. Of note, the dose of GO-101 (1 mg/kg) had absolutely no anticancer activity stand alone (data not shown), doxorubicin-sensitization at this low dose level is very encouraging. Additional studies are planned to determine doxo- or other chemo-sensitization with 5 mg/kg GO-101 daily x 21 days.

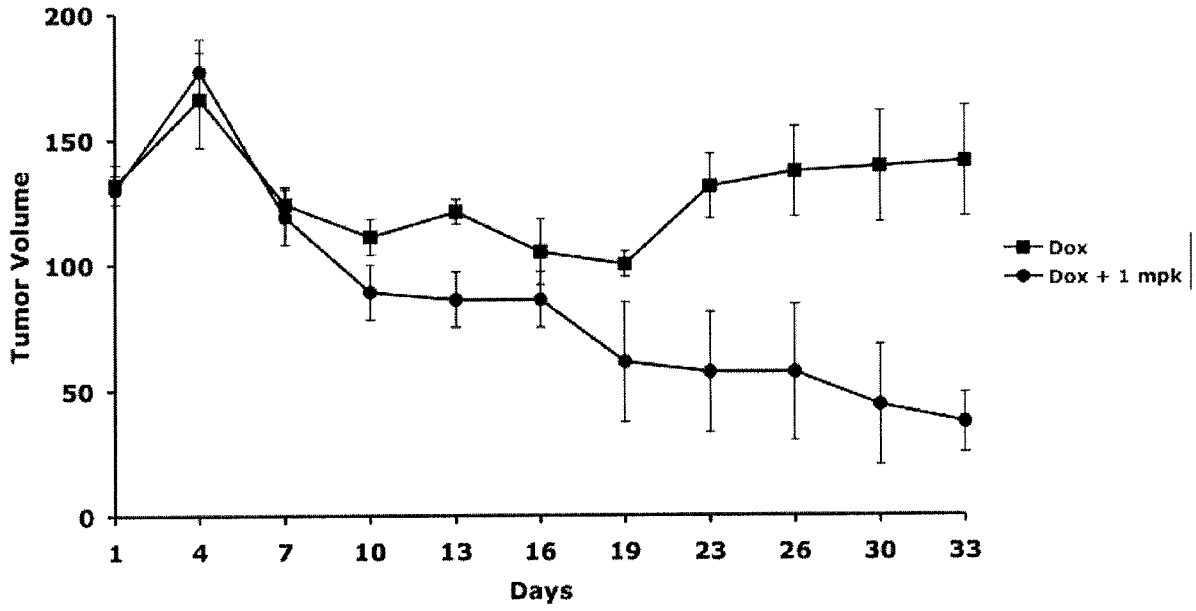


Figure 4. ZR-75-1 human breast carcinoma bearing nu/nu mice were dosed with either doxorubicin alone (squares) or in combination with 1 mg/kg GO-101 i.p daily x 21 days (circles). Tumors were measured twice a week.

8. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

10/10/11
Date

Surender Kharbanda, Ph.D.